Comprehensive transcriptomic analysis of BjGL1-knockout Brassica juncea: novel insights into leaf trichome formation and defense-associated biological processes

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Abstract

Brassica juncea is a common cruciferous crop, which can be used not only for oil extraction but also as condiments and medicinal materials. It is regarded by both traditional medicine and modern nutrition science as a food with combined dietary and health promoting value. Leaf trichomes are hair-like structures differentiated from epidermal cells and constitute an important barrier against biotic and abiotic stresses, playing a crucial role in enhancing plant resistance and thus possessing significant scientific relevance. In this study, the phenotype and gene editing site of *BjA06.GL1* and *BjB02.GL1* knockout mustard T_1 generation plants were identified. Then, RNA sequencing was performed to compare the leaf transcriptome profiles between gene-edited lines and wild-type plants, with the aim of elucidating the molecular regulatory mechanisms by which *BjGL1* controls leaf trichome development and associated biological processes in mustard. The sequencing data showed that, on average, 90.64% of the reads uniquely aligned to the *Brassica juncea* (Xuecai) reference genome. A total of 4,604 differentially expressed genes were identified in this study. Compared with the gene knockout mutant, 1,831 genes were significantly upregulated and 2,773 genes were downregulated in mustard leaves with trichomes. The differentially expressed genes were mainly enriched in pathways related to cytochrome P450 (CYP), transporters, environmental adaptation, and plant–pathogen interactions. These pathways are closely associated with secondary metabolite biosynthesis, transmembrane transport, and responses to abiotic stress and pathogen defense. qRT-PCR validation confirmed consistent expression trends of trichome regulatory genes screened from transcriptome data. This study provides an important theoretical basis for elucidating the molecular mechanisms underlying trichome-mediated resistance in mustard.

Introduction

A recent study demonstrated that simultaneous knockout of *BjA06.GL1* and *BjB02.GL1* genes resulted in the complete loss of leaf trichomes in *Brassica juncea*, leading to a significant reduction in aphid resistance (Heng et al. 2024). Leaf trichomes in *Brassica juncea* play a critical defensive role against aphids by limiting their feeding and colonization, thereby contributing to enhanced plant resistance (Kumar and Banga 2017). Trichomes function as the first line of defense in plant defense systems, protecting plants against pathogens and insect herbivores primarily through their mechanical structures and the secretion of bioactive compounds (Blaschek 2024; Nandi et al. 2022). Therefore, the development of *Brassica juncea* cultivars with enhanced leaf trichome density represents a major objective in plant breeding programs aimed at improving insect resistance.

The genus *Brassica*, one of the most economically important groups within the *Brassicaceae*, includes major vegetable crops such as Chinese cabbage, cabbage, and mustard, as well as oilseed crops such as rapeseed. Trichomes constitute the first layer of physical defense against feeding pests in Brassica crops, and trichome formation modulates glucosinolate and volatile metabolite biosynthesis, which directly determine the taste and edible quality of leafy Brassica vegetables(Xu et al. 2026). The development of aphid-resistant cultivars would reduce reliance on chemical pesticides and contribute to

more sustainable agricultural production (Zhang et al. 2024). Breeding *Brassica juncea* cultivars with high leaf trichome density can effectively protect seedlings from major pests such as diamondback moth and flea beetles, thereby reducing dependence on chemical insecticides and delivering substantial ecological and economic benefits (Heydarian et al. 2024). In *Brassica napus*, leaf trichome density has a significant influence on aphid feeding preference (Hao et al. 2019). Leaf trichomes constitute a natural physical barrier against pests and pathogens in Chinese cabbage, and they are also a key trait influencing texture and overall quality (Wei et al. 2024). Studies have shown that overexpression of *AtGL1* significantly enhances resistance of *Arabidopsis thaliana* to herbivorous insects such as flea beetles and sawflies (Sato et al. 2019). The genetic locus influencing leaf trichome traits in Chinese cabbage is located on chromosome A06, and variation in the *BrGL1* allele plays a key role in determining trichome development (Wei, et al. 2024). In Chinese cabbage, *BrrTCP4b* and *BrrTTG1* negatively regulate trichome development by suppressing the activity of the MBW complex (Li et al. 2024). The *BoTRY* gene markedly suppresses trichome formation in cabbage *(Brassica oleracea)* (Mei et al. 2017). In glabrous *Brassica napus* plants, overexpression of *BnGL3*, *BrGL3*, or *BvGL3* individually significantly increases leaf trichome density (Heydarian, et al. 2024). In radish, the two *GL1* homologs, *RsGL1a* and *RsGL1b*, can independently contribute to leaf trichome formation (Muto and Matsumoto 2022). Collectively, these studies indicate that *GL1* plays a crucial regulatory role in trichome development in *Brassicaceae* species, with its expression closely associated with trichome density and morphology. Transcriptomic analysis has shown that *PpMYB25* mediates a regulatory network involved in trichome formation in peach (Huang et al. 2025). The transcriptomes of hairy (GZZY-23) and hairless (PI246331) peppers were analyzed, and functional enrichment of differentially expressed genes revealed key DEGs associated with trichome development, hormone signaling, transcription factor families, and plant resistance (Gao et al. 2021). As a core transcriptomic platform in functional genomics, RNA-seq serves as an indispensable strategy to screen candidate genes underlying agronomic traits and diverse plant stress responses(Ali et al. 2026). However, transcriptome analyses of mustard trichomes remain limited, especially in gene-edited mustard.

Brassica juncea is an important member of the *Brassicaceae* family. Its leaf trichomes are unicellular, non-glandular, and unbranched, serving as a protective barrier against both biotic and abiotic stresses. The genetic architecture underlying trichome variation in *Brassica juncea* is complex, and structural variation in the MYB transcription factor gene *BjuVB02G54610* is associated with differences in leaf trichome phenotypes (Meng et al. 2023a). Recent studies have identified two major genes controlling trichome development in the *Brassica juncea* genome, namely *BjA06.GL1* and *BjB02.GL1* (Heng, et al. 2024). In this study, we examined the impact of CRISPR/Cas9-mediated knockout of *BjA06.GL1* and *BjB02.GL1* on leaf trichome development in *Brassica juncea*, particularly regarding trichome formation and insect resistance. Understanding the functions of these major genes will offer critical guidance for breeding trichome-rich, pest-resistant mustard varieties and promoting environmentally sustainable cultivation. CRISPR/Cas9 provides a simple and efficient approach for genetic modification in plants, while RNA-seq analysis is a powerful technique for investigating gene expression and regulatory mechanisms. In this study, we investigated the expression patterns and regulatory networks of

differentially expressed genes (DEGs) in *BjGL1* (*BjA06.GL1* and *BjB02.GL1*) knockout mustard, aiming to provide valuable insights into leaf trichome development and insect resistance.

Materials and methods

Plant materials and growth conditions

CRISPR/Cas9-mediated genome editing was employed to simultaneously target the first and third exons of *BjA06.GL1* and *BjB02.GL1*. The corresponding two guide RNAs were assembled into a binary CRISPR/Cas9 vector and introduced into *Brassica juncea* via Agrobacterium-mediated transformation, resulting in T0 genome-edited plants carrying loss-of-function mutations in both *BjA06.GL1* and *BjB02.GL1*. The self-pollinated mustard line H2, which exhibits a normal trichome phenotype, was used as the wild-type control (BT). The T_1 progeny of the *BjA06.GL1/BjB02.GL1* double-knockout lines, characterized by a trichome-deficient phenotype, were designated as BG. Both BT and BG plants were grown on half-strength Murashige and Skoog (1/2 MS) medium under controlled conditions (25°C, 16 h light / 8 h dark photoperiod). To prepare 100 mL of 1/2 MS medium, MS basal salts (a mass of 0.22 g), MES buffer (a mass of 0.05 g), and sucrose (a mass of 3.0 g) were dissolved in distilled water. Agar was supplemented at a mass of 0.7 g per 100 mL to solidify the medium. The pH was adjusted to 5.8–6.0 prior to autoclaving. The medium was then sterilized by autoclaving at 121°C for 20 min and used for plant cultivation after cooling. After 21 days of cultivation, plants were subjected to phenotypic evaluation. Leaves were collected from 21-day-old BT and BG plants and pooled within each sample. Three independent biological replicates were prepared for each genotype.

Genotype identification

Genomic DNA was extracted from the leaves of T_1 plants using the CTAB method.

PCR amplification was carried out using primers designed to flank the two target sites. PCR was performed under the following conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were purified and cloned into the pMD19-T vector, followed by transformation into *Escherichia coli*. Individual colonies were selected for Sanger sequencing. Sequencing data from three independent replicates were analyzed by sequence alignment to characterize CRISPR/Cas9-induced mutations at the target loci.

Off-target detection of gene-editing vectors

In this study, the BLAST GUI Wrapper module of TBtools was used to align sgRNA1 and sgRNA2 against the whole-genome FASTA sequences of XC to identify genomic off-target sites. Each off-target site was extended by 500 bp upstream and downstream, and the corresponding sequences were extracted using

the Fasta Extract tool in the Sequence Toolkit of TBtools. Upstream and downstream primers flanking the off-target regions were designed using Oligo 6 and synthesized by General Biosystems. The PCR program was set as follows: initial denaturation at 95°C for 5 min; 33 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; final extension at 72°C for 5 min, followed by incubation at 12°C. Genomic DNA extracted from transgenic lines was used as the template for PCR amplification. Amplified products were examined via agarose gel electrophoresis. Fragments with expected sizes were purified and ligated into the pMD19-T vector at 16°C overnight. The ligation products were transformed into *Escherichia coli*. Single colonies were picked and cultured, and positive clones were subsequently confirmed by colony PCR. Sanger sequencing was performed to verify three positive clones. Raw sequencing data were analyzed using SeqMan, and all figures and results were processed using Adobe Illustrator.

RNA-seq sequencing and transcriptome data analysis

Total RNA was extracted using TaKaRa MiniBEST Plant RNA Extraction Kit (Code No: 9769) and assessed by NanoDrop. The BT samples were designated as BT-1, BT-2, and BT-3, while the BG samples were designated as BG-1, BG-2, and BG-3. RNA was fragmented and reverse-transcribed to generate cDNA and sequencing libraries were constructed using the NEBNext Ultra RNA Library Prep Kit before sequencing on an Illumina NovaSeq 6000 platform (PE150). After removal of adaptors and low-quality reads, clean reads were quality-checked with FastQC (Roser et al. 2019). Reads were aligned to the XC reference genome using HISAT2 (Kim et al. 2015), and transcript structures were assembled with StringTie (Pertea et al. 2015).

Identification of differentially expressed genes

Gene expression levels were quantified as FPKM (fragments per kilobase of transcript per million mapped reads). Principal component analysis (PCA) was performed in R using the log2-transformed gene expression matrix. The first two principal components (PC1 and PC2) were extracted and visualized as a scatter plot with sample labels to evaluate clustering patterns among BT and BG samples. Differentially expressed genes (DEGs) between BT and BG samples were identified using DESeq2, with thresholds of $|\log2(\text{fold change})| \geq 1$ and adjusted P-value < 0.05 (Rapaport et al. 2013). Functional annotation and enrichment analyses of DEGs were performed using Gene Ontology (GO) and KEGG pathway databases to explore biological processes and metabolic pathways affected by the loss of *BjA06.GL1* and *BjB02.GL1* functions.

qPCR analysis

Reverse transcription was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (RR037A, TaKaRa, Japan) to generate first-strand cDNA. Each 20 μL reaction contained 10 μL of SYBR Green PCR mix, 0.8 μL each of forward and reverse primers (10 μM), and 8.4 μL of diluted cDNA template. Reactions

were performed on a CFX96 Real-Time PCR System (Bio-Rad, USA) under the following conditions: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s. Specificity of amplification was verified by melting curve analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, and results are presented as mean ± SEM of three biological replicates. The *BjActin* gene was used as the internal reference. Primers used in this study are listed in Table S5.

Statistical analysis

All gene expression datasets were subsequently subjected to statistical analyses via IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was first performed to examine the normal distribution of gene expression levels among all experimental groups. Levene's test was performed to evaluate homogeneity of variances. Datasets violating the homogeneity-of-variance assumption were analyzed via the two-sample t-test assuming unequal variances (Welch's t-test) for intergroup comparisons. The threshold for statistical significance was set at $P < 0.05$.

Results

Phenotypic analysis and identification of CRISPR-edited sites

Trichome development on leaves of the *BjA06.GL1* and *BjB02.GL1* double-knockout T_1 lines (BG) was compared with that of the wild-type control BT. In BT plants, abundant trichomes were observed on both the adaxial and abaxial leaf surfaces as well as at the base of the petiole (Fig. 1a, b, c), whereas the leaf surface of BG plants was smooth and completely devoid of trichomes (Fig. 1f, g, h). Scanning electron microscopy (SEM) further revealed that trichomes in BT were unicellular and unbranched (Fig. 1d, e), while no trichomes were detected on the leaves of BG plants (Fig. 1i, j).

PCR amplification of eGFP-specific primers confirmed the positive transgenic lines. Sanger sequencing of the two homologs of the *BjA06.GL1* and *BjB02.GL1* in T_1 positive transgenic plant revealed insertions and deletions at the sgRNA target sites. A 100-bp fragment containing the sgRNA1 site was deleted in the first exon of *BjA06.GL1*, resulting in a frameshift mutation, while no mutation was detected at the sgRNA2 site in the third exon. A single base (T) insertion was present at both the sgRNA1 site in the first exon and the sgRNA2 site in the third exon of *BjB02.GL1*, leading to a frameshift mutation and ultimately the loss of function of *BjGL1* (Fig. 1k). To clarify the cause of the 100-bp fragment deletion containing the sgRNA1 site, we aligned the sequences flanking the deleted fragment and found a conserved 3-bp motif (GAT) located near the breakpoints. Microhomology-mediated end joining (MMEJ) may be involved in the repair of DNA double-strand breaks, leading to frameshift mutations and loss of gene function. These results demonstrate that simultaneous knockout of *BjA06.GL1* and *BjB02.GL1* leads to the loss of leaf trichomes in *Brassica juncea*, indicating that these two homologous genes play essential roles in trichome initiation and development. Furthermore, the edited alleles were stably inherited in the T_1 generation.

To evaluate the targeting specificity of sgRNAs, we screened for potential off-target sites in the T_1 transgenic line BG. Sequence alignment analysis showed that sgRNA1 contained two base mismatches with *BjA01.XTR6* and *BjB05.XTR6*, both of which were selected as potential off-target loci. SgRNA2 contained two base mismatches at the ChrB06 locus. Further PCR amplification and sequencing validation confirmed that no insertions, deletions or substitutions were detected at the potential off-target loci targeted by sgRNA1 and sgRNA2, and the sequences of transgenic plants were completely identical to those of the wild type (Fig. S1). Collectively, neither sgRNA1 nor sgRNA2 induced off-target events. The two sgRNAs displayed high overall targeting specificity, and the gene editing system established in this study was stable and reliable.

Transcriptome profiling and differentially expressed gene analysis between BT and BG

To characterize transcriptomic differences associated with leaf trichome development, RNA-seq libraries were prepared from BT and BG plants, each represented by three biological replicates. A total of 37.68 Gb of clean sequencing data were obtained, and all samples exhibited Q30 values above 94.83%, confirming high data quality (Table 1). Clean reads were aligned to the *Brassica juncea* XC reference genome, with more than 89.49% of reads from each sample successfully mapped. Gene expression levels in leaves with trichome and leaves without trichome were calculated based on FPKM values, and differentially expressed genes were identified using a cutoff of $|\log_2$ fold change$|\geq 1$. Using BG as the reference group, 4,604 genes were found to be differentially expressed in BT, including 1,831 upregulated genes and 2,773 downregulated genes (Fig. 2a) (Table S1). Cluster analysis of significantly upregulated and downregulated genes showed that the DEGs formed distinct expression clusters between BT and BG (Fig. 2b).

Table 1
Summary of RNA-seq data

BT-1	Obtained Reads	Obtained Base (bp)	Coverage(%)	Q30 (%)	GC (%)
	23,279,854	**6,965,552,072**	**90.44%**	**94.835**	**47.90**
BT-2	19,708,202	5,900,147,764	91.33%	94.985	47.65
BT-3	21,373,434	6,399,579,314	90.80%	94.96	47.43
BG-1	20,451,348	6,122,946,336	89.49%	95.2	47.45
BG-2	21,312,426	6,376,746,840	90.85%	94.86	47.22
BG-3	19,793,702	5,924,653,872	90.90%	95.1	47.40

K-means enrichment analysis of DEGs between BT and BG

Based on the Pearson correlation coefficient ($P < 0.05$), clustering analysis was performed on 4,604 DEGs, yielding three gene clusters with significantly distinct expression patterns (Table S2). Cluster A contained 2,671 genes, which were significantly enriched in pathways including cytochrome P450 metabolism, signal transduction, and environmental information processing. Cluster B consisted of 10 genes, which were exclusively enriched in metabolic pathways. Cluster C included 139 genes, which were primarily associated with pathways such as organismal systems, environmental adaptation, and plant-pathogen interaction. These clustering results categorized large-scale DEGs into functionally related gene modules, providing clear targets for subsequent dissection of core regulatory pathways.

GO and KEGG enrichment analysis of DEGs between BT and BG

Gene Ontology (GO) analysis was performed to characterize the functional distribution of DEGs between BT and BG. In the biological process category, DEGs were predominantly enriched in response to oxygen-containing compounds, response to chemicals, response to wounding, response to stimulus, and response to external biotic stimulus. In the molecular function category, transcription regulator activity and DNA binding transcription factor activity were the most highly represented terms. In the cellular component category, DEGs were mainly associated with the cell periphery, and the plasma membrane (Fig. 3a). Collectively, these results indicate that leaf trichome development in mustard is closely associated with transcriptional regulation and stress and stimulus responsive pathways, as well as membrane-related cellular functions. To further investigate the biological pathways associated with leaf trichome formation, the DEGs were subjected to KEGG pathway annotation. The most significantly enriched pathways between BT and BG were mainly involved in environmental information processing, organismal systems, environmental adaptation, plant–pathogen interaction, and cytochrome P450–related metabolism (Fig. 3b). The cytochrome P450-related metabolic pathway catalyzes the oxidative transformation of endogenous precursors and exogenous toxic compounds in plants; this pathway mediates the biosynthesis of defensive secondary metabolites, thereby regulating plant resistance to insect pests and diverse biotic and abiotic stresses. In addition, flavonoid biosynthesis and other secondary metabolite biosynthesis, as key branches of the phenylpropanoid pathway, were significantly enriched in the DEGs, suggesting that the target genes contribute to defense responses by regulating phenylpropanoid metabolism (Fig. 3b). These results suggest that trichome development is closely linked to environmental responses and secondary metabolic processes.

Analysis of Differentially Expressed Transcription Factors Related to Trichomes

To investigate the gene regulatory networks underlying trichome formation in *Brassica juncea*, we further performed a comparative identification of transcription factors between BT and BG lines. BLAST analysis of the *Brassica juncea* genome revealed the presence of 4,391 transcription factors (Table S3). In addition, a total of 357 differentially expressed transcription factors were identified between BT and BG (Fig. 4a) (Table S4). These transcription factors were classified into more than 28 distinct families

(Fig. 4b). Among them, ERF, MYB, and NAC transcription factors represented the three most abundant families. Cluster analysis showed that these transcription factor families could be divided into three clusters based on distinct gene expression patterns, and transcription factors within the same cluster exhibited similar expression and regulatory patterns (Fig. 4c). Furthermore, the distribution of transcription factor family members across the three clusters exhibited significant heterogeneity (Fig. 4d).

A total of 299 genes related to trichome development were identified in mustard based on 80 trichome-related homologous genes from *Arabidopsis thaliana*. Chromosomal mapping was performed to determine the chromosomal locations of the 299 genes (Fig. S2). Among the 299 genes closely related to trichome development and the 357 differentially expressed transcription factors, 26 transcription factors associated with trichome development were identified (Fig. 5a). Sequence alignment identified four *GL1* homologs in the *Brassica juncea* genome. *BjA06.GL1* and *BjB02.GL1* were differentially expressed in the transcriptome, while the remaining two *GL1* homologs (*BjuLA09G063230* and *BjuLB07G024530*) exhibited no significant differential expression. Transcriptomic analysis revealed significant differences in the expression patterns of genes related to trichome development. In the BT line, the R3-type MYB transcription factor genes *BjA02.TRY (TRIPTYCHON)*, *BjA03.CPC (CAPRICE)* and *BjA09.ETC1 (ENHANCER OF TRY AND CPC 1)*, which inhibit trichome development, as well as their homologous copies, were significantly upregulated. Meanwhile, the core positive regulatory genes governing trichome formation, including *BjGL1 (GLABRA 1)* and *BjA07.GL2 (GLABRA 2)*, were also markedly upregulated in BT. In contrast, the trichome-repressing genes *BjB06.SPL5 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 5)* and *BjB06.TCP10 (TCP domain protein 10)* were downregulated (Fig. 5b). Although the expression of several trichome suppressors was elevated in BT, the strong activation of positive regulators and the repression of other inhibitory factors appear to dominate the regulatory network. The combined effects of positive and negative regulatory pathways for trichome development may contribute to enhance resistance of the BT line to aphids and other insect pests.

Verification of DEGs between leaf trichome and no trichomes mustard

To verify the accuracy and reliability of the transcriptome data, the expression levels of these genes were further validated by qRT-PCR. The selected genes included key positive regulators governing trichome growth and development, as well as negative feedback regulators and upstream repressors involved in modulating trichome density, thereby covering the core regulatory pathways underlying trichome development. Their expression patterns not only directly reflect the molecular basis of trichome morphogenesis but also are closely associated with plant defense traits including insect resistance and stress tolerance. These genes were used as validation targets to provide a rigorous assessment of the accuracy and biological significance of the transcriptome data.

Compared with the BG line, the key positive regulatory genes for trichome development, including *BjZFP8 (ZINC FINGER PROTEIN 8)*, *BjSPL9 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9)*, *BjGL2 (GLABRA2)*

and *BjMYB106 (MYB DOMAIN PROTEIN 106)*, were significantly upregulated in the BT line. The R3-type MYB transcription factor genes *BjETC1 (ENHANCER OF TRY AND CPC 1)*, *BjETC3 (ENHANCER OF TRY AND CPC 3)* and *BjCPC (CAPRICE)*, which participate in trichome patterning, were also significantly upregulated. The trichome repressor gene *BjTCP10 (TCP domain protein 10)* was markedly downregulated in BT, which relieved its inhibitory effect on trichome initiation. The coordinated transcriptional regulation of these genes promoted trichome formation and development in the BT line, and provided an important physical and molecular basis for aphid resistance (Fig. 6) (Table S6). The qRT-PCR validation results were consistent with the expression profiles obtained from transcriptome analysis, further confirming the reliability of the transcriptome data.

Discussion

Recent investigations in *Brassica juncea* have extended these findings. Two functional homologous alleles, *BjA06.GL1* and *BjB02.GL1*, have been identified, which positively modulate leaf trichome density and contribute to aphid resistance (Heng et al. 2025). Furthermore, a 3-kb structural variation (SV) at the *BjuVB02G54610* locus has been characterized as a major quantitative trait locus (QTL) underlying trichome phenotypic divergence in both natural and segregating populations (Meng et al. 2023b). Consistent with these findings, orthologous genes of *GL1* in *Raphanus sativus* are essential for leaf trichome formation. Double knockout of these two homologs led to a complete hairless phenotype (Muto and Matsumoto 2022). Collectively, these findings highlight the functional conservation and diversification of *GL1* across the *Brassicaceae*, and provide key genetic targets for breeding insect-resistant *Brassica* varieties. Nevertheless, elucidating the genetic basis of trichome development is of fundamental importance for understanding plant defense and adaptive traits.

In this study, transcriptomic profiling was performed on *BjA06.GL1* and *BjB02.GL1* gene-edited mustard mutants to advance functional genomics research in *Brassica juncea*. This approach is essential for a comprehensive dissection of the genetic networks underlying trichome development and resistance-related traits. Comparative transcriptome analysis between gene-edited plants and wild-type controls identified 4,604 differentially expressed genes, highlighting that trichome development is coordinately regulated by *BjA06.GL1* and *BjB02.GL1*. BLAST analysis identified four *GL1* homologs in the *Brassica juncea* genome. However, apart from *BjA06.GL1* and *BjB02.GL1*, the remaining two *GL1* homologs (*BjuLA09G063230* and *BjuLB07G024530*) showed no detectable transcript expression in the analyzed tissues. The expression of *GL2*, a key downstream regulator of *GL1*, was significantly altered in the *BjA06.GL1* and *BjB02.GL1* knockout lines. In *Arabidopsis thaliana*, *AtMYB5* is dispensable for trichome initiation and functions redundantly with *MYB23* to regulate trichome elongation and branching via the MBW complex (Han et al. 2022). In addition to mediating conserved trichome development, Brassicaceae *MYB5* homologs positively activate phenylpropanoid and flavonoid-dependent proanthocyanidin biosynthetic pathways, with their main regulatory effects restricted to seed coat tissues(Dai et al. 2024). Our results showed that the transcript abundance of *BjuLA01G006320.1* (*BjMYB5*) was significantly higher in BT than in BG, showing an upregulated expression pattern. In cotton, *ZFP8* is a C2H2-type zinc finger transcription factor, and its transcripts are detected in leaves and

developing fibers. Heterologous overexpression of this gene in *Arabidopsis thaliana* leads to a remarkable increase in trichome density on leaves, siliques and inflorescences (Liu et al. 2024). Consistently, *BjZFP8* exhibited significantly higher transcript levels in the trichome-rich BT line compared with the glabrous BG line. Accumulated studies in *Arabidopsis* have verified that CIN-clade *TCP10* acts as a pivotal negative regulatory transcription factor governing trichome initiation and differentiation via two synergistic regulatory routes: physical protein interaction and transcriptional activation (Lan et al. 2021). Consistently, our transcriptome data revealed that the *Brassica juncea* ortholog *BjuLB06G035350.1* (*BjTCP10*) exhibited markedly reduced transcript levels in trichome-rich BT relative to BG. This reduced expression is consistent with its conserved role as a negative regulator of trichome initiation. Importantly, our results further indicate that the functions of *BjA06.GL1* and *BjB02.GL1* extend beyond trichome formation and involve additional biological processes, particularly the phenylpropanoid biosynthesis pathway and other defense-related metabolic networks, suggesting a broader regulatory role of these two genes in coordinating epidermal development and stress adaptation. Comparative transcriptome analysis revealed extensive transcriptomic differences between BT and BG. These differentially expressed genes were significantly enriched in pathways including secondary metabolite biosynthesis, plant-pathogen interactions, and cytochrome P450 metabolism. Such large-scale transcriptomic remodeling indicates that trichome development is regulated not only by core transcription complexes but is also closely associated with plant metabolic regulation and environmental response networks(Kharechkina et al. 2023). These results provide an important reference for further dissecting the molecular mechanisms underlying trichome formation in mustard and its potential roles in plant defense.

A particularly intriguing finding of this study is the marked upregulation of numerous genes involved in secondary metabolite biosynthesis and stress responses in trichome-bearing *Brassica juncea* (BT). This result suggests a close link between trichome development and the remodeling of secondary metabolism in *Brassica*. Considering the pivotal role of cytochrome P450 family enzymes in catalyzing diverse modifications of plant secondary metabolites, such as glucosinolates, flavonoids, and terpenoids (Chakraborty et al. 2023), the differential expression of these genes likely leads to substantial differences in the metabolic profiles between trichome-deficient and trichome bearing plants in *Brassica*. In studies on glandular trichomes of *Artemisia argyi*, a combined analysis of LC-MS/GC-MS-based metabolomics and single-cell transcriptomics successfully identified 969 differentially accumulated metabolites in glandular trichomes and constructed a cell-type-specific co-expression network of sesquiterpene biosynthesis genes (Dong et al. 2026). Similarly, studies of tomato trichomes using integrated metabolomic and transcriptomic analyses revealed the regulatory mechanism of the phenylpropanoid biosynthetic pathway in insect resistance traits, and showed that the content of phenylpropanoids and the expression levels of related genes in hairy tomatoes were significantly higher than those in cultivated tomatoes (Wang et al. 2024). In addition, an integrative analysis of transcriptomics and metabolomics showed that the accumulation of flavonoids in glandular trichomes was significantly positively correlated with the increased expression of related biosynthetic genes, and identified a key transcription factor *CsTBH* regulating flavonoid biosynthesis was identified in cucumber

(Feng et al. 2023). Therefore, subsequent studies should further integrate targeted or untargeted metabolomic analyses to clarify the downstream metabolic changes resulting from these transcriptomic alterations, thereby helping to elucidate the trichome-mediated metabolic regulatory network and its potential role in environmental adaptation.

Our study provides a foundation for elucidating the molecular regulatory network governing leaf trichome development in *Brassica juncea*. By integrating transcriptome analysis with qRT-PCR validation, a series of key differentially expressed genes were identified. These genes may coordinately regulate trichome initiation and development, likely through their involvement in pathways such as secondary metabolite biosynthesis, plant-pathogen interactions, and cytochrome P450 metabolism. However, whether these transcriptomic changes directly affect the tolerance of *B. juncea* to biotic and abiotic stresses warrants further investigation. A comprehensive understanding of the trichome-mediated defense mechanism and its relationship with metabolic regulation has important implications for the molecular breeding of stress-resistant cultivars in *B. juncea*.

Conclusion

This study provides a comprehensive transcriptomic analysis of *BjA06.GL1* and *BjB02.GL1* knockout mustard, revealing their regulatory roles in trichome development and defense-associated biological processes. The identified DEG genes were mainly involved in trichome formation, plant-pathogen interaction, and cytochrome P450 metabolic pathways. These findings offer valuable insights into the genetic mechanisms underlying leaf trichome formation, with potential applications in improving insect resistance in *Brassica juncea*. Genome editing of *BjA06.GL1* and *BjB02.GL1* not only markedly affects trichome formation but also leads to broad changes in the expression of metabolism-related genes, particularly those involved in the phenylpropanoid pathway. These findings suggest that *BjA06.GL1* and *BjB02.GL1* genes may participate in border physiological processes by the regulating of secondary metabolic networks, and their precise biological functions and ecological significance warrant further investigation.

Declarations

Conflict of interest

The authors declare no competing interests.

Author Contribution

HSP and KDX conceived and designed this study; WJH, HYY and LYT performed the experiments. HSP and WJH wrote this paper; HSP, CBN, KDX and MGZ revised the manuscript; XF and CBN provided analysis tools; All the authors have read and approved the final manuscript.

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Data Availability

The RNA-seq data were deposited in the National Genomics Data Center under accession number CRA040244.

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Figures

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Figure 1

Phenotypic and gene editing site analysis of leaf trichomes in *BjA06.GL1* and *BjB02.GL1*double-knockout line of the *Brassica juncea*.

a–c. Leaf trichome phenotype of wild-type BT plants. d–e. Scanning electron microscopy (SEM) observation of leaf trichomes in BT plants. f–h. Leaf phenotype of BG double-knockout mutant plants. i–j. SEM observation of leaf trichomes in BG plants. k. Analysis of gene editing sites in *BjA06.GL1* and *BjB02.GL1*.

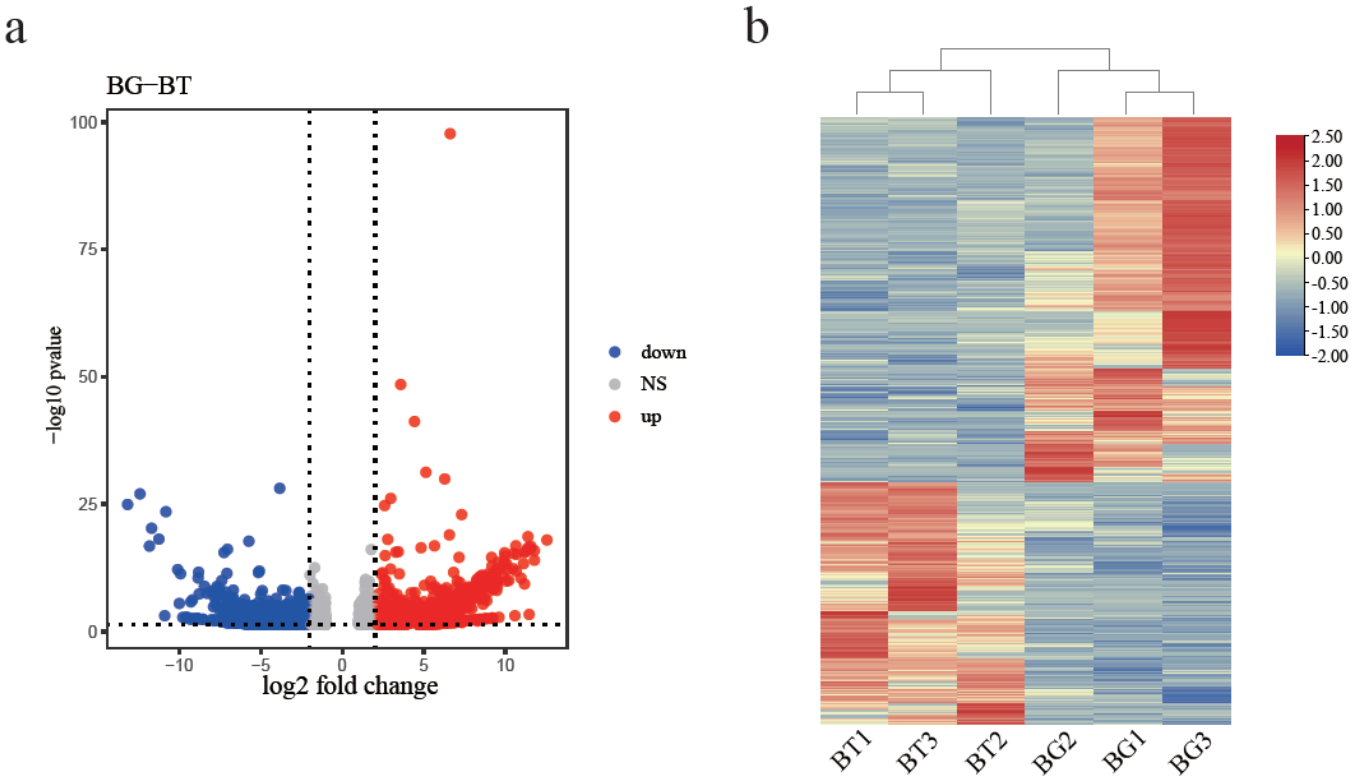

Figure 2

Transcriptome analysis and differential gene expression detection in BT and BG plants.

a. Volcano plot of differentially expressed genes between BT and BG. b. Heatmap of differentially expressed genes between BT and BG.

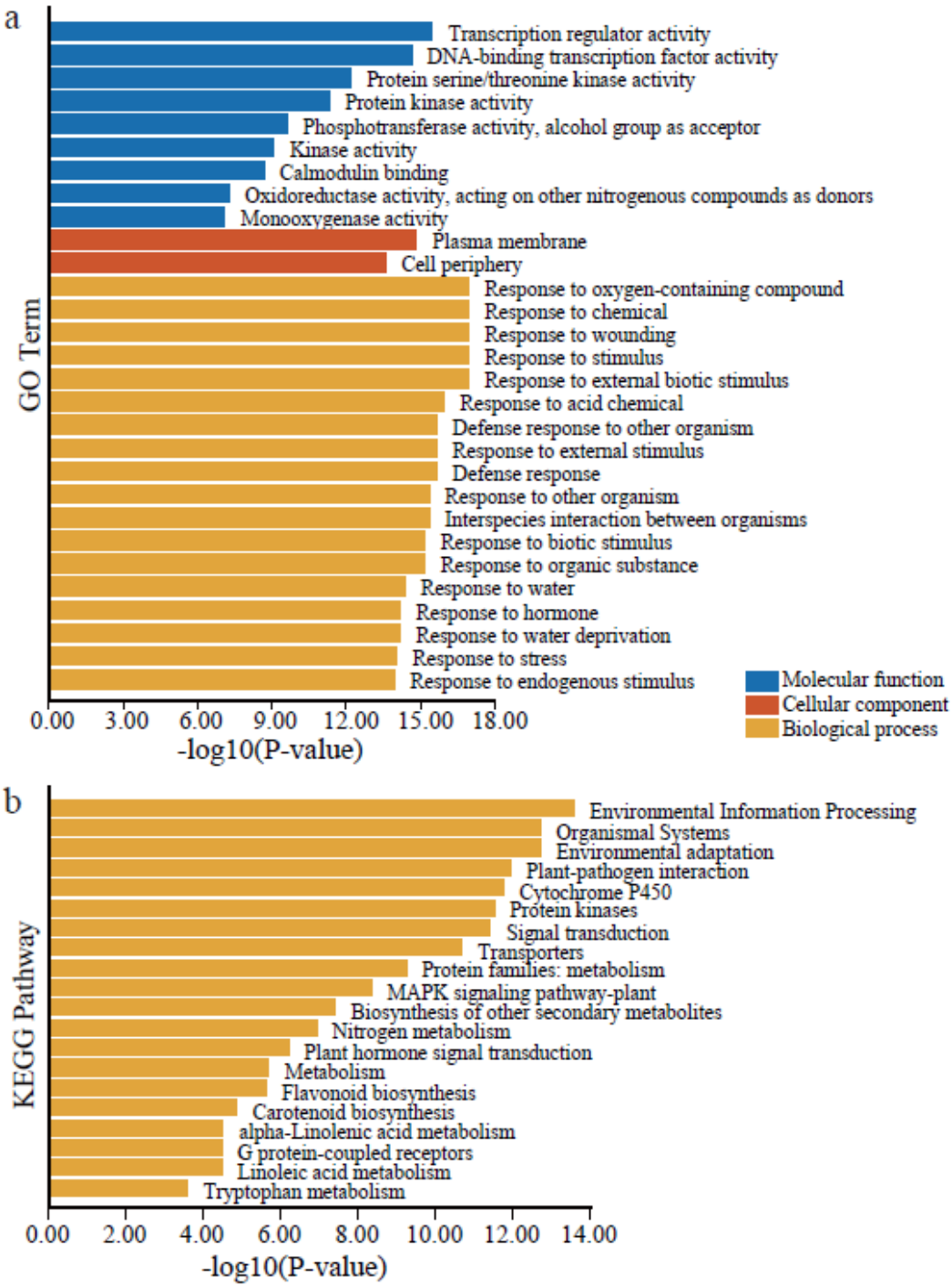

Figure 3

GO and KEGG enrichment analysis of differentially expressed genes in BT and BG plants.

a. GO functional enrichment analysis of differentially expressed genes. b. KEGG pathway enrichment analysis of differentially expressed genes.

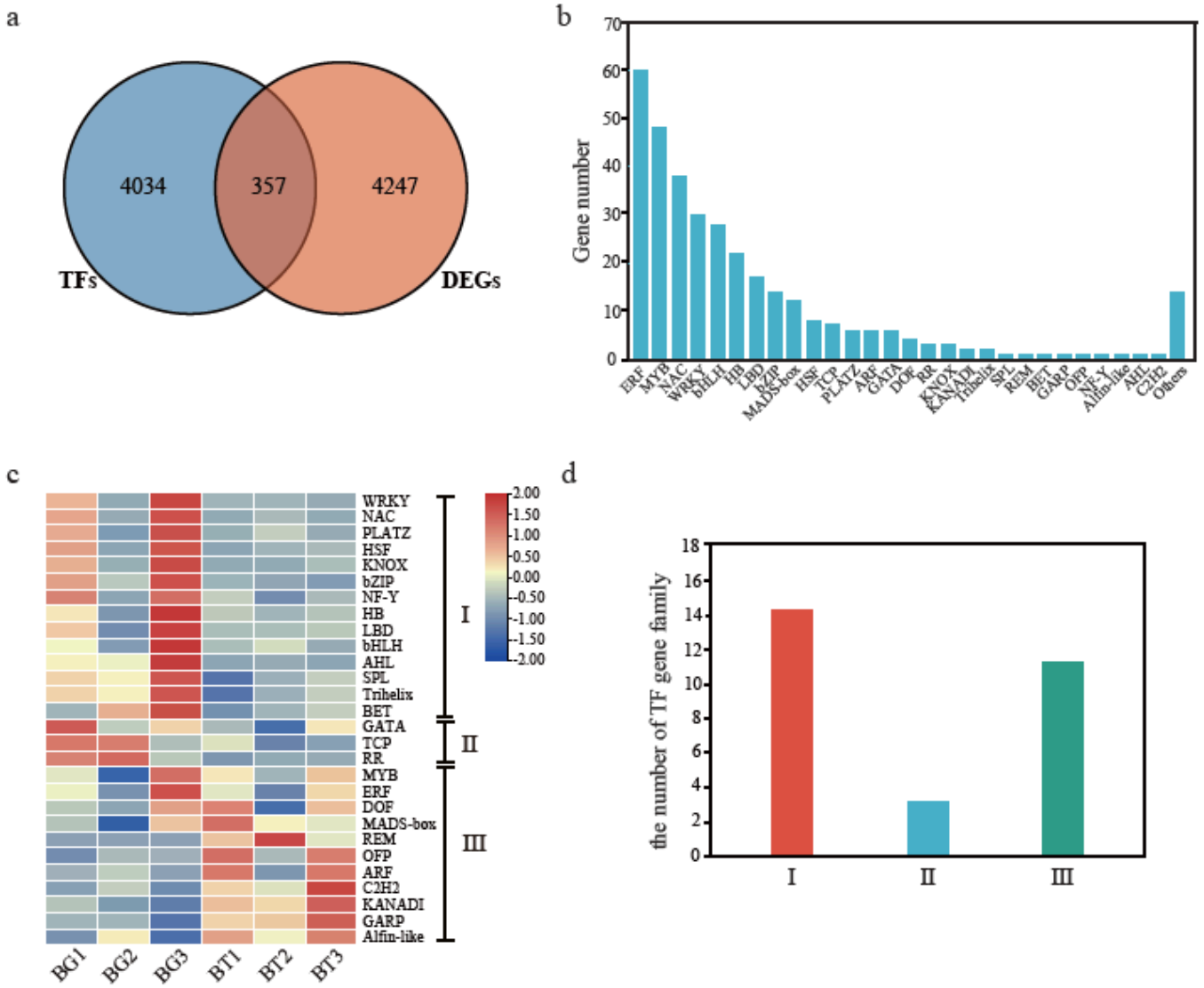

Figure 4

Differential expression pattern analysis of transcription factors.

a. Venn diagram of differentially expressed transcription factors between BT and BG. b. Distribution of gene numbers in differentially expressed transcription factor families between BT and BG. c. Cluster

analysis of differential expression patterns of transcription factors between BT and BG. d. Comparison of cluster numbers among transcription factor families.

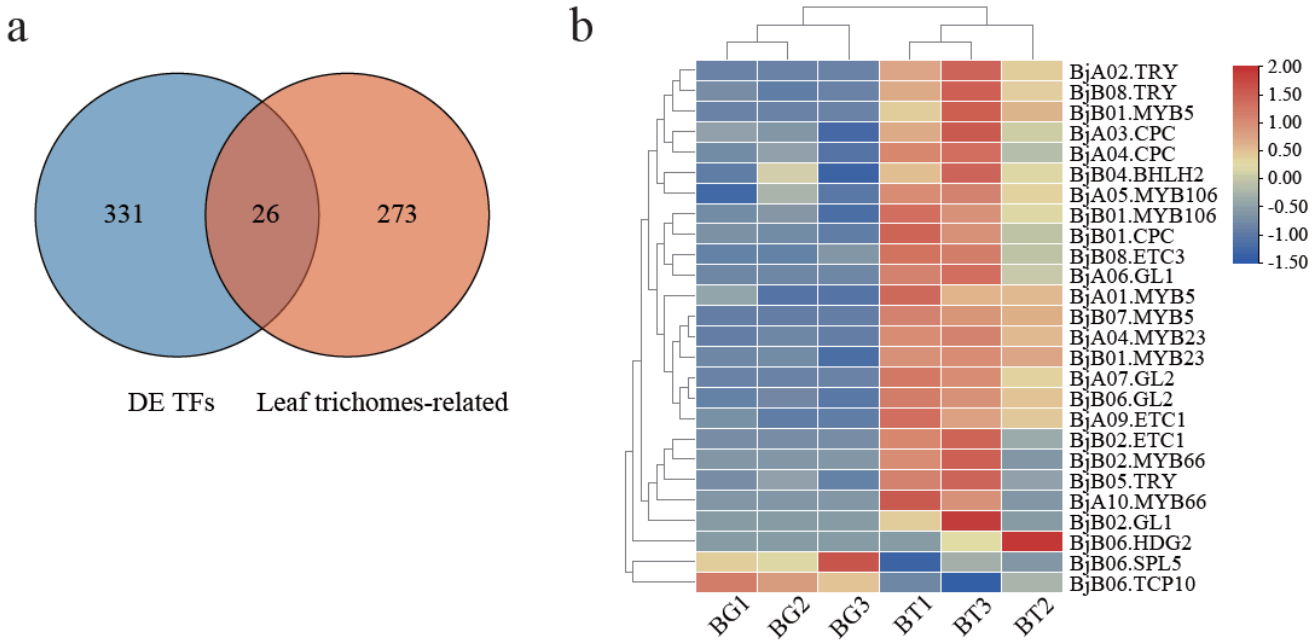

Figure 5

Analysis of differentially expressed transcription factors associated with trichome development.

a. Venn diagram of trichome related differentially expressed transcription factors. b. Heatmap of trichome related differentially expressed transcription factors.

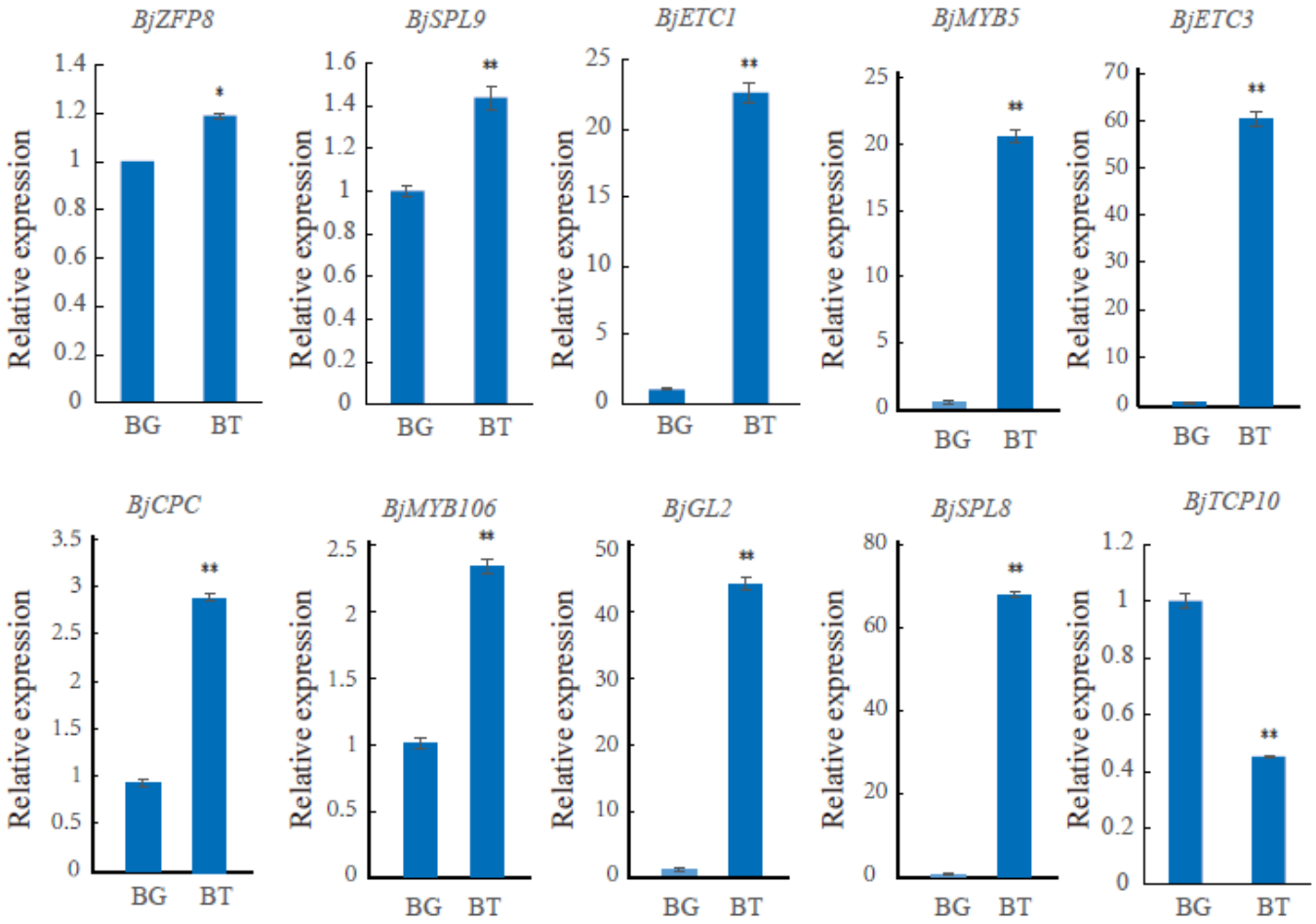

Figure 6

qRT-PCR validation of differentially expressed genes associated with trichome development.

Significant analysis was performed using BG as the control. Asterisks indicate significant differences: * $P < 0.05$, ** $P < 0.01$.

Supplementary Files

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